Population Structure and Genetic Variability of the Largehead Hairtail *Trichiurus lepturus* Linnaeus, 1758 from Indian Waters

SYNOPSIS

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(November, 2022)

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SYNOPSIS

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Date of Registration	:	22-02-2017
Place of Investigation	:	Central Marine Fisheries Research Institute, Kochi, Kerala-18, 682018

INTRODUCTION

Among diverse aquatic organisms (2, 41,327 species; according to World Register of Marine Species data retrieved October 21, 2022), paraphyletic fish represent the largest proportion of vertebrate diversity with 34,900 species (Fishbase data retrieved October 21, 2022). The general awareness about health concerns and availability of fish as a healthier and cheaper option have increased the consumption of fish in developing countries. Selective harvesting or fishing of the wild species or populations is the primary threat to the marine fishes since they can impose direct and extensive impact on the marine biodiversity and ecosystem by entirely changing the selection mechanism and population dynamics (Allendorf et al. 2008). Unlike terrestrial ecosystems, marine ecosystems have received little attention due to their vast and complex nature and sampling difficulties (Laikre et al. 2010). This calls for the need for management and conservation of fishery resources at both inter and intra-specific level so as to maintain the diversity of the harvestable resources. In order to implement efficient conservation methods, one should safeguard the processes of life since they came from the past, survive in the present and depart to the times to come. Hence the contribution from each discipline of systematic, population and evolution are unequal and irreplaceable for natural conservation (Ferrièr et al. 2004).

Deoxyribonucleic acid (DNA), one of the major macromolecule of the cell can be utilized in all the three objectives of conservation strategies because it stores the genetic information, accumulates mutations and is inherited to the next generations thereby providing historical information. Mitochondria are subcellular organelles, containing their own genetic system and involved in generation of most of the cellular ATP through the oxidative phosphorylation system (OXPHOS) (Fernández-Silva et al. 2003). Mitochondrial DNA (mtDNA) or the "second genome" is of particular interest due to its predominant female inheritance, lack or low level of recombination and paralogues, high substitution rates, high copy number and constant gene content (Cui et al. 2017). Thus, mtDNA is not just another genetic marker but forms a powerful and ideal tool to study the evolutionary history of metazoans (Avise et al. 1987). They can reveal both intra and inter-specific level sequence diversity, gene flow and selection even though the gene arrangement and functions are conserved (Yang et al. 2018).

The Largehead hairtail, *Trichiurus lepturus* (Linnaeus 1758) is considered as a valid cosmopolitan species, found in the continental shelves and slopes of temperate as well as tropical waters (114°W

- 180°E ,49°N - 54°S) (Lloris 2005). This species is benthopelagic and piscivorous which mainly feeds on small fishes, planktonic crustaceans and squids. *T. lepturus* contributes to 10-20% (11,44,000t; FAO 2022) of the overall marine fishery catch worldwide and hence become the major exploited species. In the Indian Exclusive Economic Zone (EEZ; 2.37 million km^2) *T. lepturus* forms a major fishery and has an overall annual catch of about 193822t (CMFRI 2019). Even though eight different species have been documented, this single species contributes to 95% of the total cutlassfish landings across the Indian waters (James et al. 1986; CMFRI 2019). Being a commercially important species, it is essential to exploit the fishery resource sustainably.

Not much exploited a few decades ago, *T. lepturus* is now being increasingly exploited by targeted fishery due to its export demand to Southeast Asian countries (Khan 2006). The landing of *T. lepturus* is showing an increasing trend along the Indian coasts (Joseph and Jayaprakash 2003; CMFRI 2019) and the stock is considered overexploited (Ghosh et al. 2009). In spite of the great economic and commercial significance *T. lepturus* is considered under the 'Least Concern' (LC) category by the IUCN Red list status (IUCN 2019). The species have been investigated biologically (Al-Nahdi et al. 2009), physiologically (Wilhelm-Filho et al. 2017) and ecologically (Sun et al. 2015) but not studied genetically like other pelagic fishes. Hence, the present study was designed so as to produce powerful genetic data and to provide in-depth knowledge regarding its mitochondrial genome, phylogeography, phylogeny and patterns of adaptive evolution which are essential for the development of successful fishery management programs.

OBJECTIVES OF THE STUDY

1. Genetic stock structure analysis and historical demography of the largehead hairtail, *T. lepturus* across the Indian peninsular waters using mitochondrial markers
2. Characterization of the whole mitogenome of *T. lepturus:* Insights into special characteristics and comparative mitogenomics
3. Phylogenetic considerations of *T. lepturus* using mitochondrial data with special emphasis on scombroid fishes
4. Identifying the pattern of selection in the mitochondrial protein coding genes of *T. lepturus* and teleost regional endotherms

REVIEW OF LITERATURE

Fishery genetics utilizes genetics and molecular techniques to find a solution to the taxonomical, ecological and phylogenetic disputes related to fisheries and bridge the space between geneticists and fishery managers. The use of genetic studies in fisheries research begin in 1950s (Ligny 1969) and since then the research expanded to diverse disciplines due to increase in the amount of molecular markers, decrease in expenditure, control over gene expression and enhanced knowledge about gene function (Ovenden et al. 2015). *T. lepturus* has been studied extensively across the world which provides knowledge in diverse discipline including diet and feeding habitats (Huh 1999; Martins 2005), biology (Al-Nahdi et al. 2009), taxonomic ambiguities (Tzeng et al. 2007), evolutionary effects induced by trawl selectivity (Sun et al. 2015), nutritional, storage as well as processing properties (Ren et al. 2014; Ding et al. 2019) and heavy metal accumulation (Seixas et al. 2012). Major studies of *T. lepturus* in the Indian waters concentrated on the spatio-temporal distribution (Abdul et al. 2016), resource assessment (Khan 2006), reproductive biology (Ghosh et al. 2014) and feeding habits (Mohammed et al. 2018). Several works describing the biology of this species has been done (James 1967; Pillai 1974 and James et al. 1978), which was also reviewed again (James 1973 and James et al. 1986).

1. Genetic stock structure analysis and historical demography of the largehead hairtail, *T. lepturus* across the Indian peninsular waters using mitochondrial markers

1.1. INTRODUCTION

The fisheries have extended on a global scale over the past decades due to improved technologies, introduction of motorized vessels, increased global markets and the replacement of smaller vessels with larger fleets supported by government subsidies (Pauly et al. 2002). However, if the current exploitation rate continues, the overall fisheries sector will subside by the year of 2048 (Cano et al. 2008). Hence, the need to conserve the intra-specific diversity by gaining information related to the characteristics and dynamics of that particular species so that an optimum sustainable yield can be obtained is imperative. The strategies made in every fisheries management programme should be based on the 'stock concept' which involves the identification of diversity or taxonomic groupings within a species and translation of such data for fisheries management (Carvalho and

Hauser 1995). A stock can be defined as a spawning unit, a metapopulation, a biological component or a part thereof, which can be independently exploited (Cadrin et al. 2014).

It was traditionally accepted that marine fishes show lower intra-specific genetic structuring since oceans have few dispersal barriers to obstruct the gene flow, dispersal and migration. However, several studies shows that population genetic structuring occurs in marine species due to various environmental parameters and life history traits including natal homing, selection, failure to disperse larvae across large areas and invisible oceanographic features (McLean et al. 1999; Roques et al. 2002). Increased export to Southeast Asian countries (Khan 2006), targeted fishery, the increased use of trawls (contribute to 50% ribbonfish catch in India) rather than traditional artisanal gears (Thiagarajan et al. 1992), and the accidental by catch of the species (especially in tuna long lines) (Yamandú et al. 1998) have boosted the pressure on Indian *T. lepturus* stocks which were once considered as a low value fishery (Joseph and Jayaprakash 2003). Moreover, differential exploitation of the resources among the Bay of Bengal and Arabian Sea portions of the Indian peninsula has been reported (Ghosh et al. 2014). Such practices can lead to subtle changes like reduction in genetic diversity and an increment in inbreeding at first, which on a longer run may eradicate the entire stock and productivity (Gascoigne and Lipcius 2004). Hence, a study was undertaken to delineate the genetic population structure and historical demography of *T. lepturus* with augmented taxonomic sampling covering the Indian peninsular coastal line using mitochondrial markers. Since different regions of mitochondrial DNA evolve at differential rates, two markers: Cytochrome C oxidase I (COI) and control region were used to trace out different aspects.

1.2. REVIEW OF LITERATURE

Various studies to elucidate the genetic population structure of *T. lepturus* have been done in the international waters (Hsu et al. 2007; Yoon 2013, 2014). To date, no genetic stock structure analyzes have been conducted for *T. lepturus* in Indian waters, although some stock assessment studies have been conducted (Sanjeevan 1986; Ghosh et al. 2009; Avinash et al. 2014). The demographic methods which measure extant differences among the mature individuals do not represent the dispersive larval phase, are subjective to environmental parameters (Kasapidis et al. 2008). *T. lepturus* being a neritic spawner, stock structure analysis using genetic methods rather

than demographic parameters (Martins and Haimovici 2000) can offer good results indicating the need for present study. Population genetic studies based on mitochondrial DNA amplification, sequencing and further statistical interpretation begin in the 1990s (Ferguson et al. 1995). Mitochondrial COI is commonly used as a DNA barcode (Hebert et al. 2003) or in phylogenetic studies (Saad and Abd El-Sadek 2017); however, several studies have proved its efficiency to differentiate conspecific populations owing to its slower mutation rate (Cheng et al. 2019). Due to relaxed selective pressure and decreased functional restrictions the evolutionary rate of control region is higher than any other mitochondrial genes (Hauser et al. 2001) making it more sensitive and suitable for fish population studies (Turan et al. 2015; Xiao et al. 2015).

1.3. MATERIALS AND METHODS

Specimens of *T. lepturus* were collected from five different locations (Veraval, Mumbai, Kochi, Vishakhapatnam and Puri) along the Indian coast (Fig.1). Total DNA was extracted from the muscle tissue using standard phenol chloroform extraction protocol (Sambrook and Russell 2001). Due to its hypervariability, a specific primer was designed for the amplification of control region (F115245: GATAGTCACTCCACCCCACATC, R1258: ATGTCTATCACTGCTGGGTTCC) and a universal primer (HCO2198: TAAACTTCAGGGTGACCAAAAAATCA, LCO1490: GGTCAACAAATCATAAAGATATTGG; Folmer et al. 1994) was used for amplifying the partial COI region. Genetic diversity parameters such as the number of haplotypes, haplotype diversity (h_d), and nucleotide diversity (π), number of polymorphic sites (s) and neutrality statistics (Fu's Fs, Fu 1997 & Tajima's D, Tajima 1989) were obtained in DnaSP 6.12.01 (Rozas et al. 2017). Pairwise F–statistics (Φ_{ST}) (Weir and Cockerham 1984) was determined to assess population differentiation in ARLEQUIN v3.0 (Excoffier et al. 2005). The hierarchical genetic structure was determined by Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992). To view the evolutionary relationship between haplotypes, a haplotype network was constructed via the median joining network (Bandelt et al. 1999) option in PopART version 1.7 (http://popart.otago.ac.nz). The pattern of Isolation by Distance was inferred by measuring the correlation (http://www.alcula.com/calculators/statistics/correlation-coefficient/) between genetic distance and geographic distance using the Mantel test (Rousset 1997). Mismatch distribution (Rogers and Harpending 1992) was analyzed using DnaSP 6.12.01 to check the historical population dynamics. A Bayesian Skyline Plot (BSP) was constructed to estimate past population

dynamics across time from a sample of molecular sequences, which is available in BEAST v. 1.8 (Drummond et al. 2012).

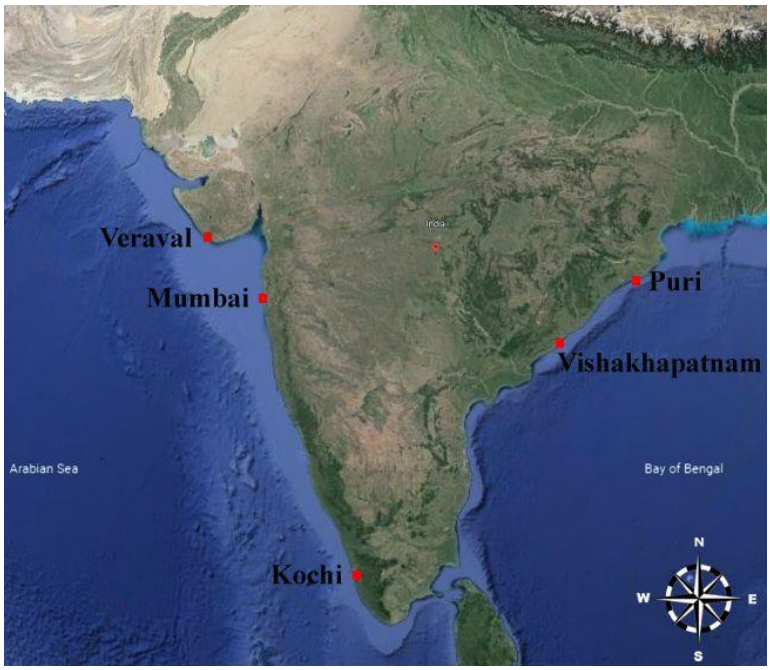

Fig. 1: Map showing sampling locations of *Trichiurus lepturus* from Indian waters

Sequences of COI (15 and 12 individuals representing East China Sea and Southwest Atlantic respectively) and control region (55 individuals representing East China Sea) were retrieved from NCBI and compared with the sequences generated in this study (Indian Ocean). The Kimura-2-parameter (K2P) (Kimura 1980) was used to calculate the genetic distance among them for the COI sequences. A phylogenetic tree was constructed to study the relationships among the samples from different oceans using the Maximum Likelihood method in MEGA v6 for both COI and control region sequences using *Eleotris acanthopoma* (AP004455.1) of the suborder Gobioidei as an outgroup.

1.4. RESULTS

A 730 bp region of the control region from 117 individuals and a 634 bp region of COI from 123 individuals (Indian Ocean) of *T. lepturus* were obtained after alignment and editing and these were deposited in GenBank with the accession numbers (CO1: MT472137-MT472166, MT476387-

MT476481; control region: MT487729-MT487757, MT499791-MT499878). The statistics for both the regions for each population are summarized in table 1.

Table 1: Genetic diversity and neutrality statistics (Fu's F_S and Tajima's D) of COI and control regions

LOCATION	N		H		h_d		π		R		S		Fu's F_s		Tajima's D	
	COI	CR	COI	CR	COI	CR	COI	CR	COI	CR	COI	CR	COI	CR	COI	CR
Veraval	15	26	6	10	0.71	0.74	0.002	0.003	1.21	2.63	7	13	-1.86	-2.47	-1.62	-0.83
Mumbai	25	22	14	9	0.89	0.65	0.003	0.006	1.40	1.39	21	36	-8.49**	-0.05*	-2.11*	-2.02*
Kochi	30	29	10	16	0.56	0.81	0.001	0.003	3.50	0.41	9	29	-8.07*	-9.05*	-1.96	-2.31**
Visakhapatnam	24	19	13	14	0.93	0.96	0.003	0.016	1.53	3.92	12	84	-7.28	-1.45*	-1.37 *	-2.16**
Puri	29	22	8	16	0.68	0.96	0.001	0.005	1.21	1.32	9	18	-3.56 *	-8.20	-1.61	-0.46

Abbreviations: Sample size (n); number of haplotypes (h); haplotype diversity (h_d); nucleotide diversity (π); Transition/Transversion bias (*R*); number of polymorphic sites (s); Statistical significance: ** P < 0.02; * P < 0.0

Among the samples, the highest pairwise Φ_{ST} values were 0.46 (Puri and Veraval) and for control region sequences and the lowest was 0.02 (Mumbai and Kochi). For the COI sequences, the highest value obtained was 0.31 (Kochi and Puri) and the lowest was 0.01 (Vishakhapatnam and Puri). The AMOVA results corroborated with the values obtained by pairwise Φ_{ST} analyses. A significant genetic differentiation was obtained when the samples was compared using control region sequences (Φ_{ST} – 0.23, P <0.001), which indicates that there is a modest population structure among populations. Similar results were obtained for COI sequences with a Φ_{ST} value of 0.12 (P <0.001. AMOVA was repeated to discover whether genetic differentiation is apparent when the populations were grouped into Arabian Sea (Veraval, Mumbai and Kochi) and Bay of Bengal (Vishakhapatnam and Puri) regions across the Indian coast. The Φ_{ST} values were significant (0.23 for control region and 0.16 for COI) with a higher variation occurring among groups (92.98 for control region and 81.30 for COI) (table 2).

Table 2: Hierarchical AMOVA test partitions the genetic variance obtained from different groupings. The results corroborated with the values obtained by pairwise Φ_{ST} analyses

Structure	Source of variation	Variance components (CR/COI)	Percentage of variation (CR/COI)	Statistics (CR/COI)	P (after Bonferroni correction) (CR/COI)
One gene pool	Among populations	0.24/0.38	76.48/ 87.47	Φ_{ST} = 0.23/ 0.12	<0.001/ <0.001
	Within populations	0.77/2.66	23.52/ 12.53		
Two gene pool: Arabian Sea (Veraval, Mumbai, Kochi) and Bay of Bengal (Vishakhapatnam, Puri)	Among groups	27.47/0.54	92.98/81.30	Φ_{CT} = -0.01/ 0.16	0.01+-0.02/ 0.02+-0.01
	Among populations within groups	0.16/0.07	0.56/2.22	Φ_{SC} = 0.24/ 0.02	<0.001/ <0.001
	Within populations	1.91/2.67	6.46/16.49	Φ_{ST} = 0.23/ 0.16	<0.001/ <0.001

Mismatch analysis revealed a unimodal plot with COI sequences (Fig. 2) indicating a scenario of recent population expansion (Ray et al. 2003) across Indian waters (Bay of Bengal and Arabian Sea waters).

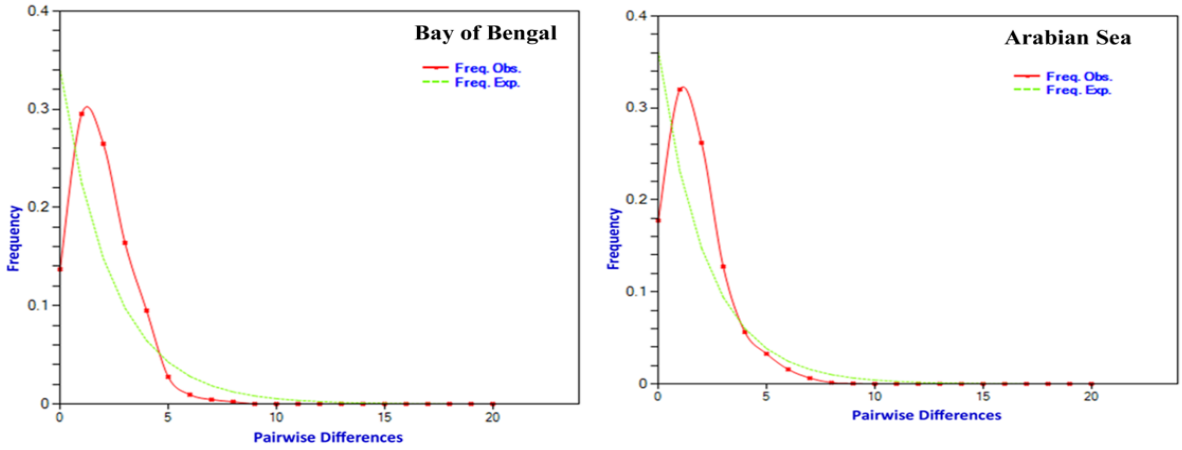

Fig. 2: Mismatch distribution plot created based on COI sequences from Bay of Bengal and Arabian Sea regions

Bayesian skyline plot revealed a population expansion for control region sequences (both Arabian Sea and Bay of Bengal) corresponding to the late Pleistocene (Fig. 3). This period experienced repeated glacial-interglacial changes and was the most recent period with punctuated glaciations (Imbrie et al. 1992). Such grave fluctuations in the climate and sea level during the late

Pleistocene have been shown to influence the population expansion (Díaz- Viloria et al. 2012) of various other marine species.

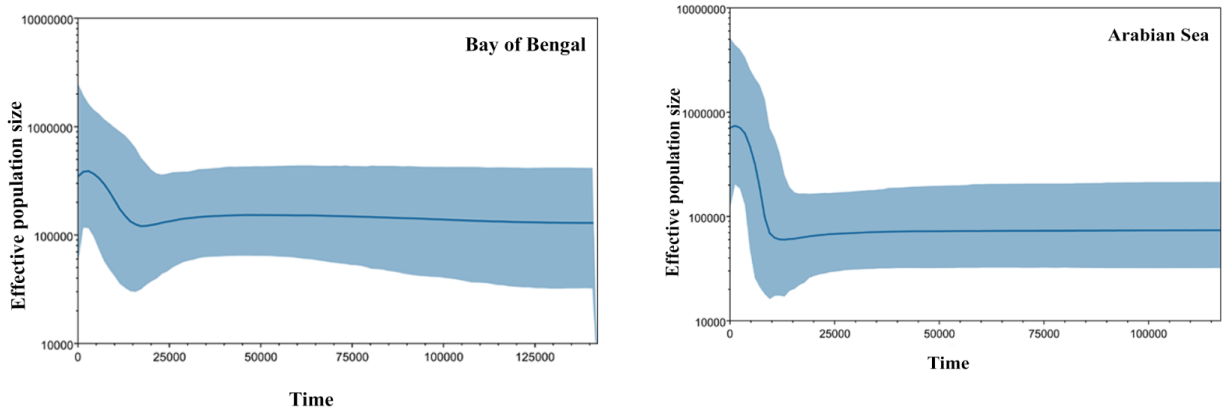

Fig. 3: Bayesian skyline plot generated using control region sequences from Bay of Bengal and Arabian Sea regions

There were four major haplogroups in the network diagram constructed using COI sequences for Indian Ocean. Out of four, two major haplogroups consisted of individuals from Kochi and Puri. The other two consisted of individuals from Vishakhapatnam and Veraval. Two major haplogroups were found to be common for all the Indian locations for the network diagram using control region sequences. The frequency of haplotypes was more on any one of the coast. The Tajima's D and Fu's Fs tests gave negative and significant values (an indication of low frequency haplotypes in excess) for both the mtDNA markers (-2.74 and -40.62 for COI; -2.57 and -32.67 respectively for control region) indicating a pattern of expanding population historically (Fu 1997). A low and insignificant ($p>0.05$) correlation ($r = 0.51$ for control region and $r = 0.46$ for COI) between $F_{ST}/(1-F_{ST})$ and geographic distance was obtained for the sequences. Thus, geographic distance alone cannot describe the way in which this species is genetically differentiated in the Indian waters.

The K2P distance inferred using COI sequences among different oceans was found to be 0.11 (Indian Ocean -East China Sea) 0.14 (Indian Ocean-Southwest Atlantic) and 0.11 (Southwest Atlantic-East China Sea), suggesting a high genetic differentiation among different marine provinces and the possibility of classifying them as separate species. The topologies of the maximum likelihood tree created using COI and control region sequences supports the K2P distance values (Fig. 4). The sequences from Southwest Atlantic, East China Sea and the Indian

Ocean formed separate distinct lineages indicating a low level of genetic flow among these populations.

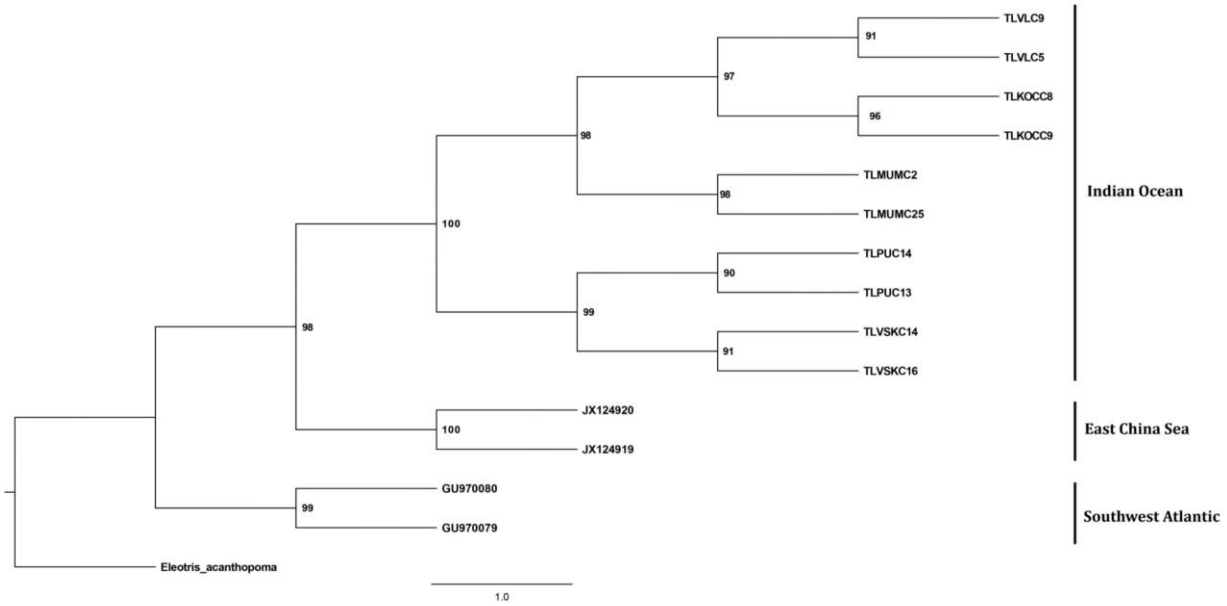

Fig. 4: Maximum likelihood tree inferred from the partial COI sequences using *Eleotris acanthopoma* as the outgroup. Haplotypes of *Trichiurus lepturus* generated in this study from various locations are used to represent the Indian Ocean: Veraval-TLVLC9, TLVLC5; Kochi-TLKOCC8, TLKOCC9; Mumbai-TLMUMC2, TLMUMC25; Puri-TLPUC14, TLPUC13; Vishakhapatnam-TLVSKC14, TLVSKC16. Accession numbers of the sequences retrieved from the database NCBI to represent East China Sea and Southwest Atlantic are also given.

1.5. DISCUSSION

The present investigation revealed a significant genetic structuring between the Arabian Sea and the Bay of Bengal as evidenced by AMOVA. The frequency distribution of each haplotype in the haplotype network diagram was more on either east or west coast confirming the results from AMOVA. The presence of a star like pattern radiating from the major haplotypes indicates that *T. lepturus* populations have experienced a recent population expansion and an increase in effective population size in this area. Bayesian skyline plot also supported a population expansion across the Arabian Sea and Bay of Bengal corresponding to the late Pleistocene period after a population bottleneck (Zhang et al. 2019).

Due to the open nature of the marine habitat, isolation by distance is considered as the main mechanism by which intra-specific differentiation can occur (Palumbi 1994). However,

geographic distance cannot explain the way in which *T. lepturus* is genetically differentiated in Indian waters due to the low and insignificant results obtained in the Mantel test. The significant genetic structuring obtained in the present study cannot be justified by a single cause but by various environmental and life-history factors including lower probability of the cross-basin migration by the adults and the juveniles, retention of the larvae in inshore areas, isolation by time, pattern of ocean currents and homing behavior which limit the gene flow among the *T. lepurus* populations.

High genetic differentiation obtained between *T. lepturus* samples from the Indian Ocean, East China Sea and Southwestern Atlantic region can be attributed to several biogeographical barriers in the open Ocean. They shape the distribution of species by restricting/limiting the larval dispersal due to the vast distance among the habitats (Gaither et al. 2010). The Indo-Pacific barrier (IPB) separates the Indian and Pacific Oceans and becomes a major biogeographical barrier that restricts the dispersal (Voris 2000). The European/African and American continents, Benguela current at the tip of Southwest Africa and the closure of the Isthmus of Panama makes the Atlantic Ocean a closed system preventing the gene flow (Floeter et al. 2008). The combined effect of all these biogeographical barriers is responsible for the genetic differentiation of *T. lepturus* across different Oceans obtained in this study.

2. Characterization of the whole mitogenome of *T. lepturus:* Insights into special characteristics and comparative mitogenomics

2.1. INTRODUCTION

Mitogenomic sequence data serves as a rich source of information for identifying new fish species and to resolve fish phylogenies. Additionally, mitogenomes can be applied towards accurate identification of fishes up to species level especially in closely related species where morphological similarities result in taxonomic ambiguities (Zhuang et al. 2013). To date, complete mitogenome of only two species have been sequenced from this genus. Hence, we sequenced the complete mitogenome of *T. lepturus*, analyzed the gene content and organization in relation to other species and the molecular mechanism that might have shaped their evolutionary dynamics. Mitochondrial genomes were thought to have fixed gene content but now have been proven to

undergo continuous gene loss events during their path of evolution (Adams and Palmer 2003). Mitogenomes range from types that have different numbers of tRNA (e.g., missing one tRNA in the *Didelphis virginiana*) to the most extreme situations (e.g., in *Trypanosoma brucei*) where the mitogenomes can be entirely devoid of tRNA genes (Salinas-Giegé et al. 2015). Here we also discuss the unusual features of the *Trichiurus* mitogenomes including tRNA loss and special characteristics of origin of L-strand replication (O_L).

2.2. REVIEW OF LITERATURE

Traditionally DNA sequencing was done by cloning followed by restriction enzyme digestion, but the method was laborious and time consuming. The availability of PCR primers was limited, since Cytb and the two rRNA genes (12S and 16S) were the most commonly sequenced genes even though 37 structural genes and non coding regions are found in mitochondria. This made the sequencing of longer mitochondrial DNA even more strenuous (Kocher et al. 1989; Miya and Nishida 2015). Complete mitogenome sequencing was first brought into the limelight by Miya and Nishida during the end of the 20th century and since then an 'era of mitogenome' was born (Broughton 2010). They used a novel PCR based method in which a blend of long PCR procedure together with versatile fish PCR primers was performed (Miya and Nishida 1999). This method can be applied to wide range of teleosts and is much faster than sequencing of cloned mitochondrial DNA. Since then, complete mitochondrial genome of several freshwater and marine species has been sequenced which led to the revolutionization of the fish mitogenomics (Inoue et al. 2000; Broughton et al. 2001; Kim et al. 2004). To date, complete mitogenome of only two species have been sequenced from the genus *Trichiurus; T. japonicus, T. nanhaiensis* (Liu and Cui 2009 and Liu et al. 2013).

2.3. MATERIALS AND METHODS

Initial amplifications of the partial segments of mitochondrial COI, 16SrRNA, ND2, Cytb and ATPase 6/8 genes (in the size range of 500bp – 800bp) of *T. lepturus* were carried out by five sets of universal primers. Additional primers were further designed using Primer3 (Untergasser et al. 2012) for amplifying contiguous, overlapping segments of the complete mitogenome of *T. lepturus*. Bidirectional sequencing of the amplified products was conducted by an ABI 3730 automated sequencer. The position of the 13 protein coding genes (PCGs) and two rRNAs was

determined by using MitoFish MitoAnnotator (Iwasaki et al. 2013). The genome map was constructed by the use of OGDRAW (Lohse et al. 2013). The codon usage of the 13 PCGs was analysed with MEGA v6 (Tamura et al. 2013). The AT-skew [(A−T)/(A+T)] and GC-skew [(G−C)/(G+C)] values were used to describe the strand symmetry or overall patterns of nucleotide composition (Perna and Kocher 1995). Most of the tRNA genes were identified by tRNAscan-SE1.21 (Lowe and Eddy 1997) and ARWEN (Laslett and Canback 2008). The putative O_L was identified with RNA Structure (Reuter and Mathews 2010) and Mfold Web Server (Zuker 2003), the tandem repeats in control region were analyzed using Tandem Repeat Finder v3.21 (Benson 1999).

2.4. RESULTS

The complete mitogenome sequence of *T. lepturus* is a 16,840bp closed circular molecule which contain the canonical 13 PCGs (COI-III, ND1-6, ND4L, ATP6, ATP8 and Cytb), 2 ribosomal RNA genes (12S and 16S) and two non-coding regions (O_L and the control region). The mitogenome has only 21 tRNA genes except for $tRNA^{Pro}$. The nucleotide frequency in the complete mtDNA was found to be A:28.3%, T:25.9%, C:29.4% and G:16.2%. Most of the genes were encoded on the heavy strand (H-strand), except for ND6 and seven tRNA genes (Fig. 5, Table 3).

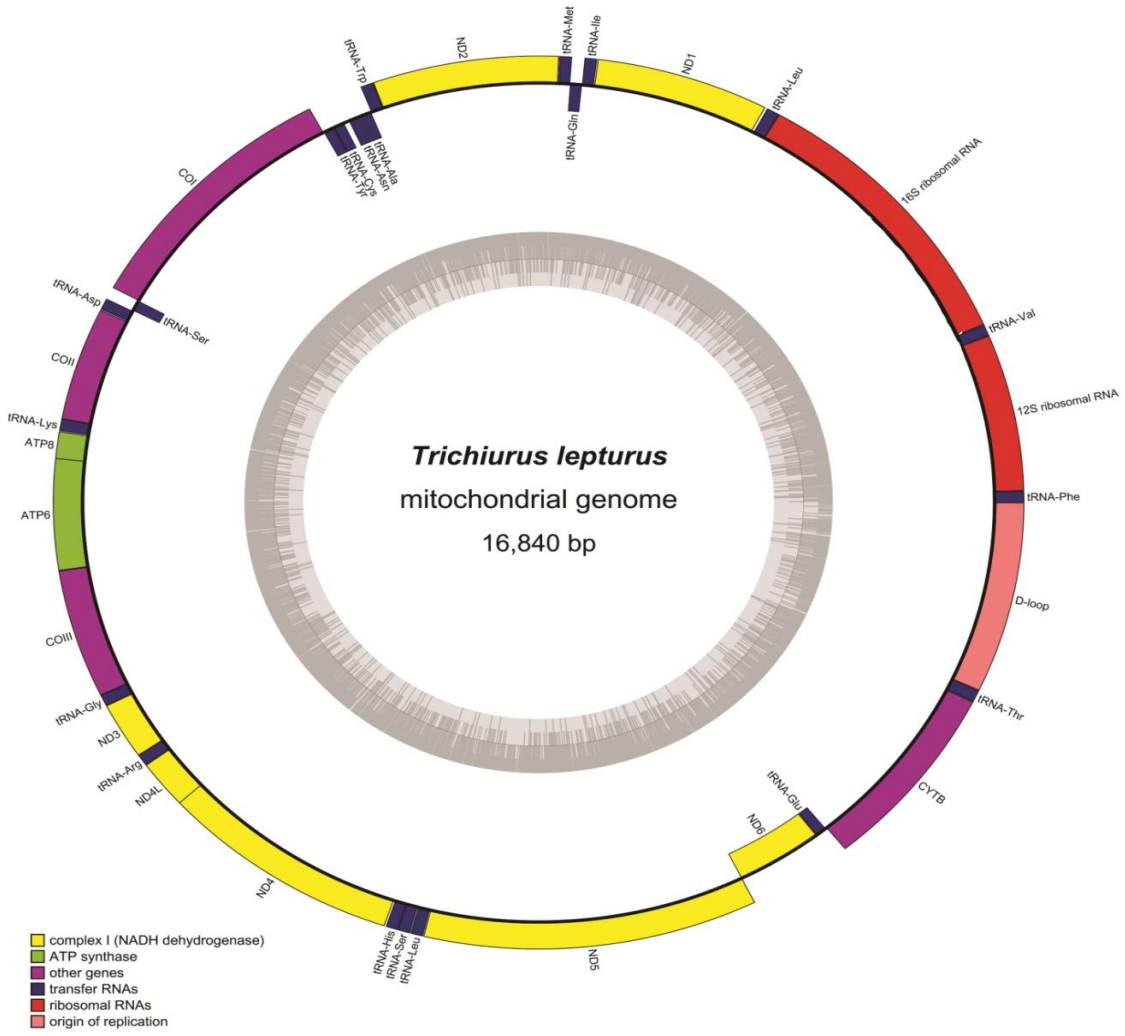

Fig. 5: Gene content and organization of the largehead hairtail *T. lepturus* mitochondrial genome (GenBank accession number: MK333401). Genes coded by the heavy and light strand are shown outside and inside of the ring, respectively. The GC content is shown by the inner grey ring.

Table 3: Characteristics of the mitogenome of *Trichiurus lepturus*

GENE	POSITION		SIZE	CODON		STRAND	tRNA	Spacer
	FROM	TO	(bp)	START	STOP		ANTICODON	(+)/Overlap (–)
$tRNA^{Phe}$	1	69	69			H	GAA	0
12S rRNA	70	1026	957			H		0
$tRNA^{Val}$	1027	1097	71			H	TAC	0

16S rRNA	1098	2822	1725			H		0
$tRNA^{Leu}$	2823	2896	74			H	TAA	11
ND1	2908	3882	975	TTA	TAA	H		3
$tRNA^{Ile}$	3886	3955	70			H	GAT	-1
$tRNA^{Gln}$	3955	4025	71			L	TTG	-1
$tRNA^{Met}$	4025	4095	71			H	CAT	1
ND2	4097	5142	1046	ATG	TAa	H		0
$tRNA^{Trp}$	5143	5216	74			H	TCA	1
$tRNA^{Ala}$	5218	5286	69			L	TGC	1
$tRNA^{Asn}$	5288	5360	73			L	GTT	31
O_L						L		
$tRNA^{Cys}$	5392	5457	66			L	GCA	0
$tRNA^{Tyr}$	5458	5524	67			L	GTA	1
COI	5526	7076	1551	GTG	TAA	H		0
$tRNA^{Ser}$	7077	7147	71			L	TGA	2
$tRNA^{Asp}$	7150	7222	73			H	GTC	2
COII	7225	7915	691	ATG	Taa	H		0
$tRNA^{Lys}$	7916	7988	73			H	TTT	2
ATPase8	7991	8158	168	ATG	TAA	H		-8
ATPase6	8149	8832	684	ATG	TAA	H		-1
COIII	8832	9616	785	ATG	TAa	H		0
$tRNA^{Gly}$	9617	9685	69			H	TCC	0
ND3	9686	10034	349	ATT	Taa	H		0
$tRNA^{Arg}$	10035	10103	69			H	TCG	0
ND4L	10104	10400	297	ATG	TAA	H		-5
ND4	10394	11764	1371	ATG	AGA	H		7
$tRNA^{His}$	11772	11840	69			H	GTG	0
$tRNA^{Ser}$	11841	11911	71			H	GCT	2
$tRNA^{Leu}$	11914	11985	72			H	TAG	2

ND5	11988	13868	1881	ATG	TAA	H		-2
ND6	13865	14386	522	ATG	TAG	L		0
$tRNA^{Glu}$	14387	14455	69			L	TTC	4
Cyt b	14460	15600	1141	ATG	Taa	H		0
$tRNA^{Thr}$	15601	15674	74			H	TGT	0
Control Region	15675	16840	1166			H		

The mitogenome of *T. lepturus* contained 21 tRNA genes varying in size from 66bp to 74bp. Three tRNA clusters (IQM, HSL, and WANCY) were conserved in *T. lepturus* as in other vertebrate mitogenomes. Two types of anticodons (TGA, GCT) determined serine and TAG and TAA determined leucine. A positive AT skew (0.1036) and a negative GC skew (-0.1096) were observed, showing a bias towards As and Cs when horizontal alignment of 21 tRNAs was performed. An asymmetry in the AT and GC content of the light and heavy tRNAs was also observed. tRNAs on the heavy strand had positive AT skew (0.1029) and a negative GC skew (-0.0505) values indicating a low G content. Whereas, those on the light strand had negative AT skew (-0.1082) and positive GT skew (0.2307) values, indicating high G content. The smaller 12S rRNA subunit and the larger 16S rRNA subunit have a size of 957bp and 1725bp, respectively and are located on the heavy strand. Both the segments are separated by $tRNA^{Val}$ and flanked on either side by $tRNA^{Phe}$ and $tRNA^{Leu}$. The A: C: G: T base composition of the 16S and 12S rRNAs gene is 32%, 28.1%, 19.6% and 20.4%. Skew values of rRNAs showed a similar pattern to that of tRNA genes with an AT skew value of 0.2213 and a GC skew value of -0.1781. This indicates that A and C content is more prevalent in mitochondrial RNA genes.

There are two long non-coding regions in the mitochondrial genome of *T. leptururs*: Origin of L-strand replication (O_L) and control region, which are important in the transcription, replication and maintenance of the mitogenome (Fernández-Silva et al. 2003). The O_L is found in the domain between $tRNA^{Asn}$ and $tRNA^{Cys}$ located in a cluster of five tRNA genes (WANCY). The conserved motif 5'-GCCGG-3' involved in the transition from RNA synthesis to DNA synthesis is modified (5'-ACCGG-3') and is found at the base of the folded hairpin structure within the $tRNA^{Cys}$ gene. Moreover, the loop which initiates RNA primer synthesis is shorter (4 bp) which is normally in the

range of 10-14bp in other bony fishes (Wei et al. 2013). The control region found between $tRNA^{Thr}$ and $tRNA^{Phe}$, had a length of 1166bp and an A+T content of 67.1% which is higher than the average value of whole genome (54.3%), a common feature reported in vertebrates with a negative AT skew (-0.0342) and GC skew (-0.1124) values (strongly biased towards T and C). Eight perfect and three imperfect tandem repeats of 15bp was identified which might have originated via slipped-strand mispairing during mtDNA replication (Levinson and Gutman 1987).

The 13 PCGs in the mitogenomes of *T. lepturus* were similar to those of other vertebrates and include 7 NADH ubiquinone oxidoreductase subunits (ND1-6, ND4L), 3 cytochrome c oxidase subunits (COI–III), 2 ATP synthase subunits (ATP6 and ATP8*)* and a cytochrome b oxidoreductase subunit (Cyt b). The PCGs were strongly biased against G (32.5%A; 35.8% C; 9.7% G; 22% T) at the third codon position, which was typical in vertebrate mitochondrial genomes (Inoue et al. 2001). The most common nucleotide at the second codon position was T (40 %) and pyrimidines were over-represented (68.4 %), owing to the strong hydrophobic character of proteins (Naylor et al. 1995). However, at first codon position, purines are slightly higher than pyrimidines. The codon usage of genes oriented in opposite direction shows strand asymmetry due to nucleotide composition. A clear preference for C in the wobble position of H strand encoded genes can be seen (33.1%A; 37.1% C; 8.6% G; 21% T), whereas T or G ending codons are represented in ND6 (19.5%A; 6.9% C; 32.2% G; 41% T), which is encoded by the L-strand.

Total number of codons used in 13 PCGs was found to be 3983 excluding the stop codons. Leucine 1 (Leu 1, 437), and Alanine (Ala, 370) are the most common and cytosine (Cys, 34), the least common aminoacid. The amino acids Leu and Ser were used by six different codons, while all other amino acids were encoded by either two or four. Relative Synonymous Codon Usage (RSCU) values greater than 1 indicate that those codons are more commonly used and vice versa. Pro (CCG, 0.21) and Ala (GCG, 0.21) occur rarely and Arg (CGA, 2.47) occur more frequent (Fig. 6).

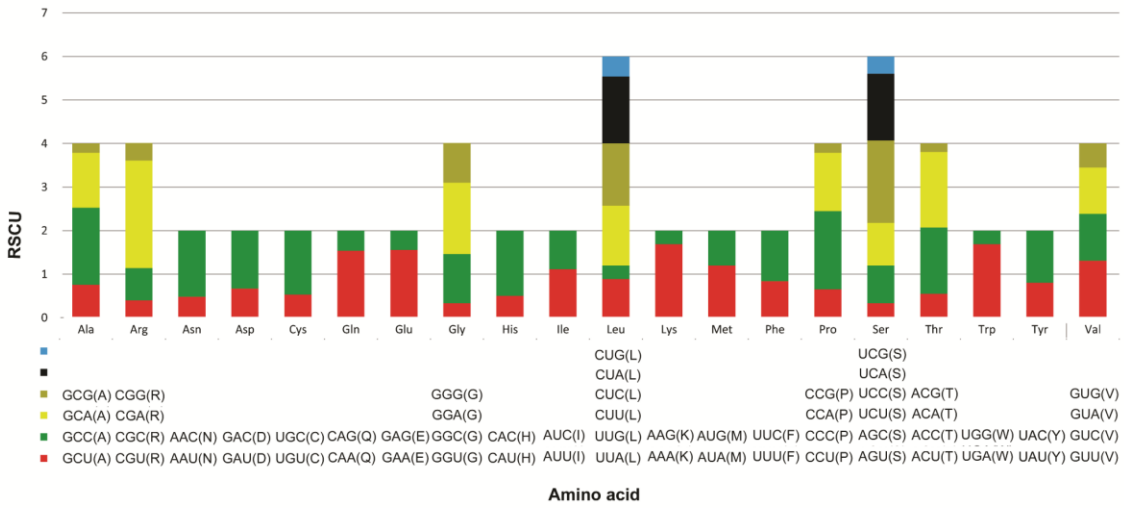

Fig. 6: Relative Synonymous Codon Usage (RSCU) in the mitogenome of *T. lepturus*

Table 4: Outline of the base composition of mitochondrial genomes of the concatenated 13 protein-coding genes (PCGs) at each codon position across seven species of the family Trichiuridae.

Species	Accession number	Size (bp)	Whole genome composition						
			A%	T%	G%	C%	A+T%	AT Skew	GC Skew
Trichiurus lepturus	MK333401	16840	28.3	26	16.2	29.5	54.3	0.04235	-0.2910
Trichiurus japonicus	EU339148.1	16796	28.7	25.8	16.2	29.3	54.5	0.0532	-0.2879
Trichiurus nanhaiensis	NC 018791.1	17060	28.5	26	16.2	29.3	54.5	0.0458	-0.2879
Aphanopus carbo	AP012944.1	16406	27.74	24.68	16.68	30.87	52.42	0.0583	-0.2984
Benthodesmus tenius	AP012522.1	16864	26.67	24.43	17.24	31.64	51.1	0.0438	-0.2945
Assurger anzac	AP012508.1	16510	26.30	24.90	17.46	31.32	51.2	0.0273	-0.2841
Evoxymetopon poeyi	AP012509.1	16475	27.69	25.59	16.24	30.46	53.28	0.0394	-0.3044

2.5. DISCUSSION

The complete mitogenome sequence of *T. lepturus* was deposited in the GenBank database under the accession number MK333401. No sequences were found elsewhere in the mitochondrial genome showing similarity to the missing $tRNA^{Pro}$ which challenges the traditional opinion of fixed gene content for vertebrate. The $tRNA^{Pro}$ gene is also absent in other two *Trichiurus* (Liu

and Cui 2009 and Liu et al. 2013) species making it a common feature among the genus. Since no template was found in the mt genome for extensive RNA editing for reforming a ‘‘tRNA-Pro’’ transcript to a functional $tRNA^{Pro}$, the translational function in mitochondria is accomplished by import of a nuclear encoded tRNA, which does a double duty to meet the needs of both cytoplasmic and mitochondrial protein synthesis. $tRNA^{Ser}$ has no discernible DHU (Dihydrouridine) stem and loop and hence cannot be folded into a typical cloverleaf secondary structure which is similar to other bony fishes (Wei et al. 2013). This $tRNA^{Ser}$ has been previously described as a minimal tRNA but can adjust the mutual distance between the 3′-terminus and the anticodon making it a functional one similar to that of usual tRNAs in the ribosome (Ohtsuki et al. 2002).

The two unusual features of $O_{L,}$ observed indicates that it may not be functional in *T. lepturus* mitogenome and is similar to *T. japonicus* and *T. nanhaiensis* (Liu and Cui 2009 and Liu et al. 2013). But the enzyme that recognizes stem and loop structure of O_L is homologous to the amino acyl tRNA synthetases that recognizes the loop formed by tRNA genes and hence initiation of DNA replication can occur at tRNA genes flanking O_L (Seligmann 2010). The asymmetry observed among the base composition of the H and L strands of the PCGs may be due to the existence of an asymmetrical directional mutation pressure. This is related to the asymmetrical replication of mtDNA leaving H-strand in a single stranded state which is more prone to oxidative and hydrolytic damages and therefore is more prone to mutations (Reyes et al. 1998).

3. Phylogenetic considerations of *T. lepturus* using mitochondrial data with special emphasis on scombroid fishes

3.1. INTRODUCTION

A robust understanding of the phylogenetic relationships among lineages is essential to understand the evolution of morphological and physiological characters. *T. lepturus* belongs to the suborder Scombroidei which also comprises Tunas, Bonitos, Mackerels, and Billfishes contributing significantly to pelagic biodiversity. They constitute both regional ectothermic and endothermic groups, with metabolic and muscular specializations for elevated aerobic capabilities, reduction of heat loss, and fast swimming. The three families of the suborder, Scombridae, Xiphiidae, and Istiophoridae, are high performance fishes, and display regional endothermy (elevation of

temperature in selected tissues) (Dickson and Graham 2004) but others do not. Despite these shared traits, the phylogenetic classification of Scombroids remains under debate, which confounds conservation strategies for this important teleost group. Previous molecular studies to infer the relationship among billfishes and scombroids were based on single locus or partial gene segments or with less number of loci. Molecular phylogeny that examines single locus does not reveal the definite evolutionary relationships of species due to possible selection at loci (Miya and Nishida 2000). Hence it is essential to carry out a study with extensive taxonomic sampling and other informative sites from longer DNA sequences (e.g., complete mitogenomes) to accurately represent the highly complex patterns of diversification of Scombroidei. Subsequently we tried to resolve the intra and inter relationships of scombroid taxa using PCGs, 12S and 16S rDNA sequences.

3.2. REVIEW OF LITERATURE

Regan (1909) originally proposed a definition of the Scombroids based on three principal osteological characters: epiotics split by the supraoccipital, beaklike premaxillae and gill membranes devoid of the isthmus (Carpenter et al. 1995). Since then, multiple alternative arrangements have been suggested. Morphological (Nakamura 1983) and molecular (Miya et al. 2013) data were used to resolve the intra- and inter-relationships of the suborder producing competing hypothesis due to the taxonomic limits of the suborder. Nelson (2006) concluded that Scombroidei comprises six families, Gempylidae, Xiphiidae, Trichiuridae, Scombridae, Sphyraenidae, and Istiophoridae, with 47 genera and 147 species. A general perception that included billfishes to Scombroidei (scombroid hyphothesis) is due to their shared morphological (hypurostegy, intricately connected gill filament blades, a beak-like structure formed by ethmoid-rostral cartilage-premaxilla complex), and physiological (regional endothermy and swimming capabilities) similarities in particular to scombrids (tunas). However, the scombroid hypothesis is now being challenged by the molecular data (Santini et al. 2013) which suggests the placement of billfishes and scombroids as separate suborders.

3.3. MATERIALS AND METHODS

Complete mitochondrial sequence of *T. lepturus* was compared with 52 teleosts including representatives of six classical Scombroidei families (Gempylidae, Xiphiidae, Trichiuridae,

Scombridae, Sphyraenidae, and Istiophoridae) (Nelson 2006), retrieved from NCBI GenBank. The *Callorhinchus milii* (Chimaeriformes) mitochondrial genome (HM147137.1) was used as the outgroup. Initially datasets for the 13 PCGSs (excluding stop codons), 12SrDNA and 16SrDNA were created and aligned separately with the Clustal X v1.81 algorithm. The final concatenated matrix of 13,025bp was used to find the optimal partition strategy and best-fit evolutionary model for each partition, with the PartitionFinder v1.1.1 (Lanfear et al. 2017). The maximum likelihood analysis was carried out in RAxML v.8.2.10 (Stamatakis 2014) with the GTRCAT model for each partition and the support for nodes were analyzed with 10000 nonparametric bootstrapping replicates. Partitioned bayesian phylogenetic inference with mixed models was carried out in MrBayes 3.2.7 (Ronquist and Huelsenbeck 2003).

3.4. RESULTS

T. lepturus, the species for which the complete mitochondrial sequence was generated in this study belongs to the Scombrodei family Trichiuridae and showed close relationship with *T. nanhaiensis* and *T. japonicus*. Our study does not support the monophyly of Scombroidei, because billfishes form a separate clade from the other scombroids, which contradicts Johnson's (1986) classical Scombroidei based on morphological characters. The phylogenies created by Bayesian and Maximum likelihood analyses were congruent and could resolve two clades, (i) Gempylidae, Scombridae, Trichiuridae, Scombrolabracidae (*Scombrolabrax heterolepis*) and Stromateoids and (ii) Xiphiidae, Istiophoridae, and Sphyraenidae. The resolution is in congruent with the pioneering work of Betancur et al. (2013) in which clade 1 is recognised as Scombriformes and clade 2 as Istiophoriformes (Fig. 7).

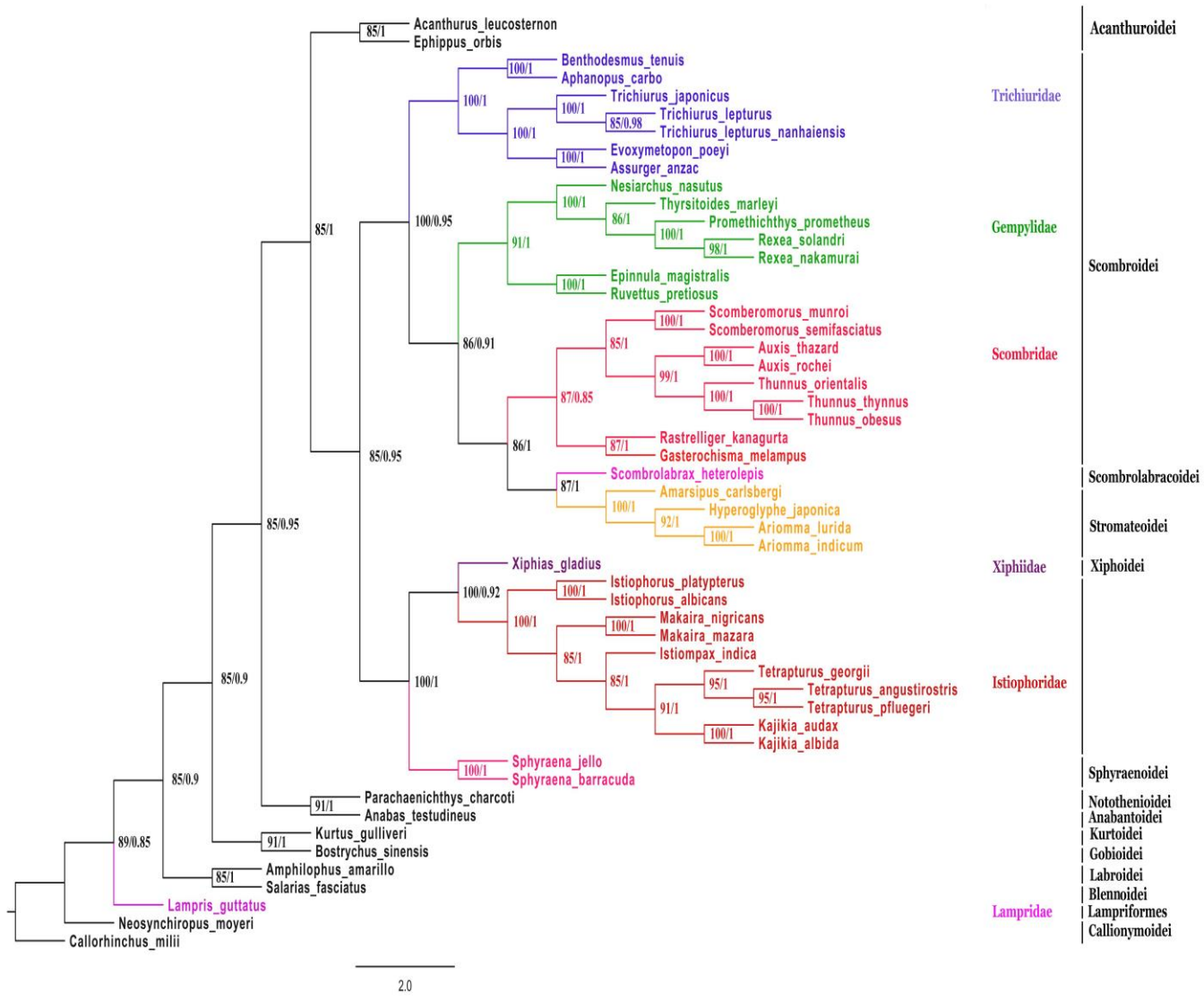

Fig. 7: Phylogenetic tree obtained from the concatenated sequences of 13 coding regions and two rDNAs (16SrDNA and 12SrDNA) of 52 teleost species with *Callorhinchus milii* as the outgroup. Numerals at the nodes indicate the bootstrap/posterior probability values. Mitochondrial genome of *Trichiurus lepturus* was generated in this study.

3.5. DISCUSSION

Our molecular phylogeny refutes the scombroid hypothesis and suggests the placement of billfishes in a separate suborder, Xiphioidei (Orrell et al. 2006; Collette et al. 2006). Due to the inclusion of several other percoid species between the billfishes and scombroids they are distant enough to reject the scombroid sister hypothesis (Finnerty and Block 1995) in which billfishes and scombroids are considered as separate sister suborders. Regional endothermy has been reported in

other teleosts also (*Gasterochisma melampus*, Scombridae and *Lampris guttatus*, Lampridiformes). Our molecular phylogenetic analysis shows that *G. melampus, L. guttatus* are not closely related to billfishes and tunas (Chen et al. 2003). Taken together, regional endothermy has evolved independently in at least four groups of teleost fishes (opah, billfish, butterfly mackerel and tunas) that do not share a recent common ancestor. Therefore, it can be concluded that endothermy evolved convergently in distant teleost groups at least at the phenotypical level in response to various selective pressures or functional requirements (Legendre and Davesne 2020).

4. Identifying the pattern of selection in in the mitochondrial protein coding genes of *T. lepturus* and teleost regional endotherms

4.1. INTRODUCTION

Mitochondrial oxidative phosphorylation genes (OXPHOS) play critical role in energy metabolism, aerobic potential and thermogenesis. The evolution of mitochondrial genome under the assumption of neutrality is now being increasingly questioned suggesting that the genetic variation of the mitochondrial DNA is not solely shaped by random genetic drift. Organisms that have encountered selective forces to improve their metabolic potential or adapt to cooler environment can be suitable candidates to study the pattern and impact of selection on mitochondrial genome. Tunas (Scombridae), butterfly mackerel (*Gasterochisma melampus*, Scombridae), billfish (Istiophoridae and Xiphiidae) and opah (*Lampris guttatus*, Lampridiformes) are the only teleost fishes to exhibit regional endothermy (Legendre and Davesne 2020). They might have experienced strong selective forces to enhance their metabolic potential making them a suitable candidate group to search for positive selection.

Given the significant roles of OXPHOS genes in energy production and thermoregulation, it is plausible that any amino acid change may influence the protein function causing significant fitness and metabolic consequences in the species (Ballard and Pichaud 2014). Hence, we hypothesize that variations in energy requirements might have induced selection pressures on the mitochondrial OXPHOS genes so that they can meet the metabolic demand and adapt to the new environment. In this study, we take the benefit of complete mitogenome sequences of teleost regional endotherms that are deposited in the GenBank and make an attempt to analyze the pattern

of selection experienced by the OXPHOS genes during the course of evolution. The pattern and the level of selective pressures imposed on different PCGs of *Trichiurus* species were also analyzed.

4.2. REVIEW OF LITERATURE

Regional endothermy is well documented in mammals and birds (McNab 1978; Lovegrove 2017) and it remained overlooked until Davy (1837) recorded elevated body temperature than the ambient waters in skipjack tunas. Several advantages for regional endothery have been propsed including enhanced vision and neural activity (cranial edothermy, Block and Carey 1985), deep cold-water foraging (Stoehr et al. 2020), higher digestion and absorption rate (Goldman 1997) and rapid recuperation from anaerobic burst (Brill 1996). However, the most accepted advantage of the trait is thought to be increased swimming performance and ecological niche expansion which might have enabled the endothermic teleosts for large scale migration (Dickson and Graham 2004; Watanabe et al. 2015).

In spite of the functional constraints, the mitochondrial PCGs are susceptible to selection and adaptation in accordance to the environmental conditions like high temperature (Morales et al. 2016), low temperature (Cheviron et al. 2014;), reduced oxygen level or hypoxia (Scott et al. 2011), availability of nutrients (Da Fonseca et al. 2008), high energy demands for flight (Shen et al. 2010), variation in gene expression (Mishmar et al. 2003) and high altitude (Yu et al. 2011) in diverse organisms including humans. Even though marine organisms are less studied unlike other terrestrial organisms, patterns of selection in mitochondrial DNA have been reported in several marine species in response to various factors like sea surface temperature (Deng et al. 2019), temperature clines (Grant et al. 2006; Silva et al. 2014), latitudinal clines (Garvin et al. 2011) and osmotic environment (Brennan et al. 2016).

4.3. MATERIALS AND METHODS

To analyse evolutionary adaptation, the rates of non synonymous (Ka) and synonymous (Ks) substitutions in the mtDNA among three species of Trichiuridae were estimated with DnaSP 6.12.01 (Rozas et al. 2017). The complete mitogenome sequences of 16 endothermic teleosts representing four families (Xiphiidae, Istiophoridae, Scombridae and Lampridae) (Johnson 1986;

Nelson 2006) were retrieved from NCBI GenBank. Initially datasets for 13 PCGs were made, aligned separately, edited manually and concatenated excluding the stop codons with the Clustal X v1.81 algorithm. The phylogenetic tree reconstructed using the maximum likelihood method was used along with the sequence alignment in further analysis to infer the sites undergoing positive selection using Mixed effects model of evolution (MEME), Fixed Effects Likelihood (FEL) and Fast, Unconstrained Bayesian Approximation for Inferring Selection (FUBAR) implemented in DATAMONKEY (http://www.datamonkey.org/). TreeSAAP was used to analyze the significant changes caused by the replacements in the physicochemical properties of the amino acids using different models under neutral conditions. (Woolley et al. 2003). Finally, the construction of 3D homology model of protein subunits was done using the SWISS-MODEL server (Schwede et al. 2003) using the best hit templates.

4.4. RESULTS

In all 13 PCGs, the Ka/Ks ratio was much less than 1 and varied from 0.0109 (COI between *T. lepturus* and *T. nanhaiensis*) to 0.2745 (ND6 between *T. japonicus* and *T. lepturus*) (Fig. 8).

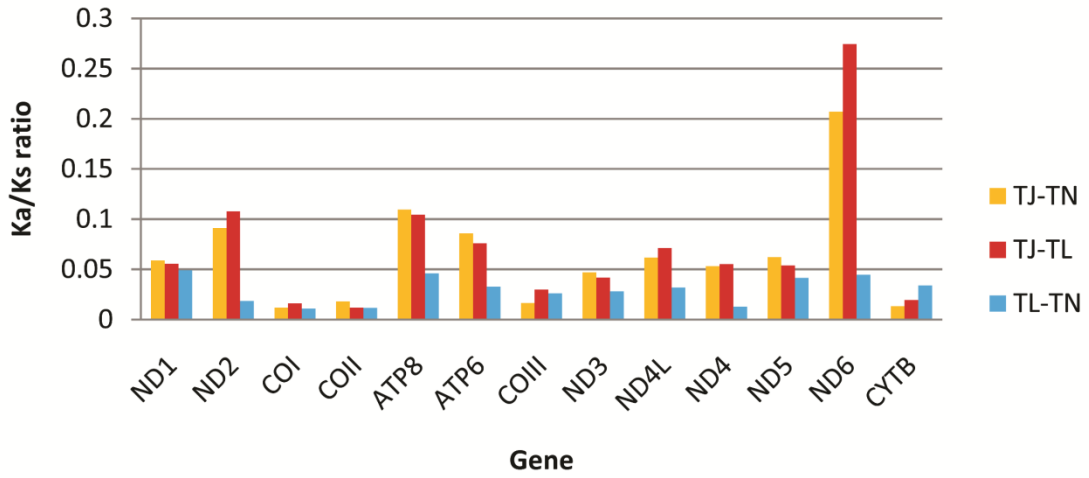

Fig. 8: Evolutionary rates or the rates of non-synonymous substitutions to the rate of synonymous substitutions (Ka/Ks) for each PCG of the mitochondrial genomes of *T. lepturus* (TL), *T. japonicus* (TJ) and *T. nanhaiensis* (TN)

Among the 3793 sites tested, MEME analysis found evidence for episodic positive or diversifying selection at 30 sites. FEL found evidence of pervasive positive or diversifying selection at 4 sites and pervasive purifying selection at 2808 sites. FUBAR found evidence of episodic positive selection at 2 sites and episodic purifying selection at 3461 sites. TreeSAAP detected 12

significant radical changes in physicochemical properties of amino acids in the genes COI (3), ATP8 (3), ATP6 (1), COIII (1) and ND5 (4). Eleven sites; COI (#262, #481), ATP8 (#51), ATP6 (#2), COIII (#50), ND4 (#446), ND5 (#17, #337, #604), ND6 (#95, #103) which showed evidence for positive selection by more than any one of the four approaches used (MEME, FEL, FUBAR, TreeSAAP) were selected for further analysis to avoid false positives (table 5). However, seven sites among them were private and found only in the nodes leading to a single individual. Hence, the significance of these terminal mutations is unable to verify. Four positively selected sites were found to occur at the basal region of a particular lineage leading to multiple individuals and therefore are likely to play an important role in the evolution and adaptation of the species. Among them three were found in the complex I (NADH dehydrogenase): (i) two sites in ND5 (#17 and #604) at the base of the branch leading to the suborder Xiphoidei and endothermic tunas respectively and (ii) one site in ND6 (#103) at the base of the branch leading to the entire suborder Scombroidei and Xiphoidei (Fig. 9, 10). A significant mutation in the gene ATP6 (#2) was found at the base of the branch leading to the family Istiophoridae (Fig. 11). These sites were then located in the three-dimensional structure of the protein subunit predicted using appropriate templates. The best hit homology protein subunits or templates for each gene were: ND5 (SMTL ID: 6zkb.1.B), ND6 (SMTL ID: 6qc5.1.U) and ATP6 (SMTL ID: 6zpo.1.U).

Table 5: Outline of the results obtained from various tests conducted on the OXPHOS genes of sixteen regionally endothermic teleosts to detect signals of positive selection.

Gene	Amino acid position	From codon to codon	From amino acid to amino acid	MEME	FEL	FUBAR	TreeSAAP	Distribution of amino acid replacements across lineages
				P value	P value	Posterior probability	Physicochemical properties (Category)	
ND1	245	GCC-ACC	Ala-Thr	0.1				*Makaira nigricans*
ND2	220	CTC-ATT	Leu-Ile	0				*Tetrapturus angustirostris*
COI	21	GTA-CTG	Val-Leu	0				*Tetrapturus pfluegeri*
COI	117	GGG-GCC	Gly-Ala	0.06				*Tetrapturus pfluegeri*

COI	262	TCA-GCC	Ser-Ala	0.09			Coil tendencies (6)	*Thunnus thynnus*
COI	331	GCT-TCT	Ala-Ser	0.01				Istiophoridae
COI	338	CTT-ATA	Leu-Met	0.08				*Tetrapturus pfluegeri*
COI	390	GTT-ATG	Val-Met	0.09				*Thunnus thynnus*
COI	416	GTA-CTC	Val-Leu	0.06				*Tetrapturus pfluegeri*
COI	449	CTA-GTC	Leu-Val	0.1				*Tetrapturus pfluegeri*
COI	481	GAA-TTA	Glu-Leu	0.05			Chromatographic index (8), Hydropathy (8)	*Tetrapturus pfluegeri*
COI	507	GAG-CAG	Glu-Gln	0.1				Xiphoidei
ATP8	47	CCA-ACA	Pro-Thr	0.05				Xiphoidei
ATP8	51	AAC-CCC	Asn-Pro	0.03			Compressibility (6), Power to be at the C-terminal (6), Thermodynamic transfer hydrophobicity (6)	*Gasterochisma melampus*
ATP6	2	ACA-GTA	Thr-Val	0.08			Solvent accessible reduction ratio (7)	Istiophoridae
ATP6	112	GCA-GTA	Ala-Val	0.07				*Kajikia albida*
COIII	50	CTT-CCT	Leu-Pro	0.09			Compressibility (6)	*Lampris guttatus*
COIII	98	TTC-TTA	Phe-Leu	0.04				*Istiophorus* albicans
COIII	121	ATT-CTA	Ile-Leu	0.09				Tunas
COIII	175	TTC-CTC	Phe-Leu	0.07				*Kajikia albida*
ND4L	24	ACC-TAC	Thr-Tyr	0.01				Xiphoidei
ND4	422	GCA-CAC	Ala-His	0.08				*Lampris guttatus*

ND4	446	ATC-GCC	Ile-Ala	0.04	0.094			*Gasterochisma melampus*
ND5	17	ACA-TCA	Thr-Ser	0.08	0.084			Xiphoidei
ND5	337	ACT-CCT	Thr-Pro	0.07			Compressibility (6)	*Lampris guttatus*
ND5	404	GCC-ACC	Ala-Thr	0.08				Xiphoidei + Scombroidei
ND5	538	ACA-GCA	Thr-Ala	0.01				Xiphoidei + Scombroidei
ND5	604	ACC-GCC	Thr-Ala	0.05			Hydropathy (6), Solvent accessible reduction ratio (8), Surrounding hydrophobicity (8)	Tunas
ND6	11	GGT-TCT	Gly-Ser	0.03				Xiphoidei
ND6	95	ATG-CTG	Met-Leu	0.01	0.024	0.901		*Gasterochisma melampus*
ND6	103	GTG-GCG	Val-Ala	0.03	0.021	0.96		Xiphoidei + Scombroidei

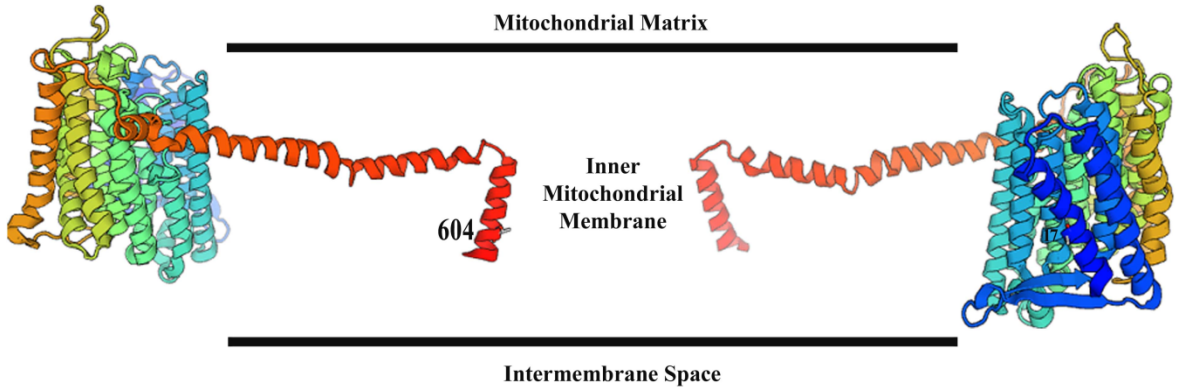

Fig. 9: Three-dimensional representation of the mitochondrial subunit ND5 of respiratory complex I created using SWISS-MODEL. Site showing positive selection is marked in bold.

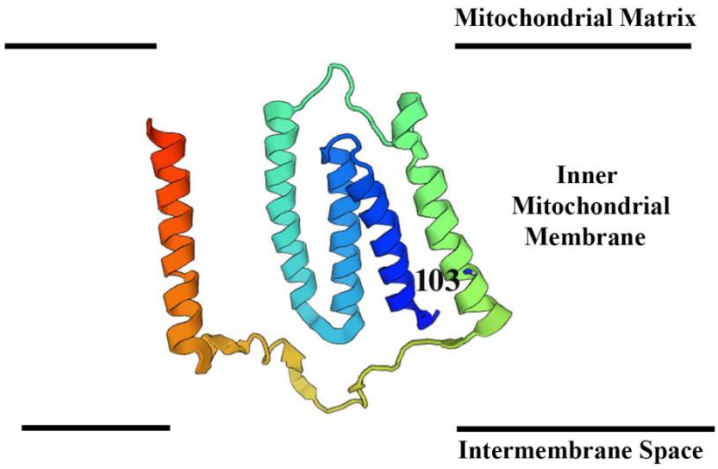

Fig. 10: Three-dimensional representation of the mitochondrial subunit ND6 of respiratory complex I created using SWISS-MODEL. Site showing positive selection is marked in bold.

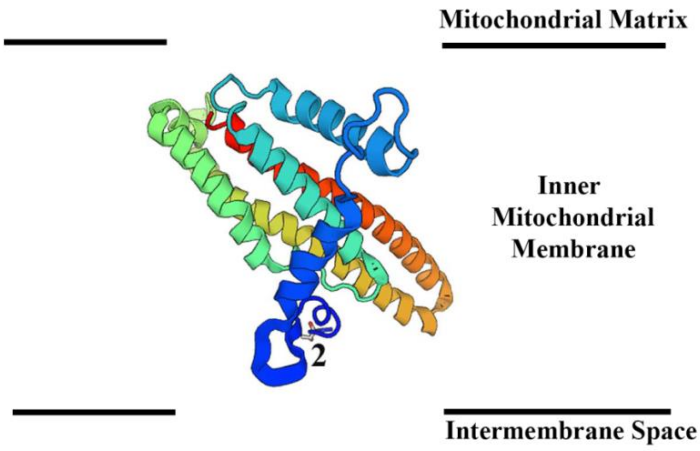

Fig. 11: Three-dimensional representation of the mitochondrial subunit ATP6 of complex V (ATP synthase) created using SWISS-MODEL. Site showing positive selection is marked in bold.

4.5. DISCUSSION

We calculated the ratio of the rate of non-synonymous substitutions (Ka) to the rate of synonymous substitutions (Ks), in order to analyze the level of selective pressures imposed on different protein-coding genes of *Trichiurus* species. The ratio (ω) partitions the targeted sequences into three basic scenarios: negative (purifying) selection if Ka < Ks (ω < 1), positive (adaptive) selection if Ka > Ks (ω > 1), and neutral mutation if Ka = Ks (ω = 1) (Zhang et al.

2006). The results indicate that all the PCGs were evolving under purifying selection which means natural selection has been taking place against deleterious mutations resulting in negative selective coefficients. Evolutionary pressures to conserve the ancestral state of the protein result in purifying selection coefficients (Yang and Bielawski 2000). The average Ka/Ks ratio differs substantially among individual genes, indicating that different genes are likely to evolve in different ways due to varying functional constraints. Additionally, the highest ratios were in ND6 and ATP8 in the L- and H-strand, respectively, indicating that the selection pressures acting on both strands were independent.

We found evidence for positive selection in different mitochondrial protein subunits across several branches of the phylogeny of the teleost regional endotherms. Among the 13 PCGs, ND genes encoding the subunits of the mitochondrial complex I harbored highest number of candidate sites under positive selection. They are placed immediately upstream to the origin of light strand replication (O_L) and/or downstream to the origin of heavy strand replication (O_H). During the process of replication these genes remain single stranded for much more time when compared to the other mitochondrial genes and hence they are vulnerable to high mutation rate (Marshall et al. 2008).

The sites that exhibited signatures for positive selection in the mitochondrial complex I (ND5 and ND6) are located in the transmembrane helix. The subunits ND2 and ND4 are joined together by the ND5 arm facilitating a coordinated switch in proton pumping (Hunte et al. 2010). ND6 plays an important role in the assembly and arrangement of the complex I by forming salt bridges and hydrogen bonds to their neighboring subunits (Efremov et al. 2010). The site which showed evidence for positive selection in the ATP6 is located in the speculated internal helix loop region. ATP6 is a subunit of the membrane inserted F_0 proton channel of the complex V (ATP synthase) and plays an important role in the rotary catalysis of ATP (Spikes et al. 2020). Even though we could not identify the direct relationship of the positive selections detected to the functional implications and fitness consequences on the species, we could conclude that these variations can improve the overall ATP production which could theoretically augment the heat production and metabolic potential of the individual. Temperature is one of the chief selective force acting on mitochondrial DNA (Ballard and Whitlock 2004) and positive selection on mitogenome have been

known to increase the aerobic capacity (Grossman et al. 2004) and thus adaptation to low temperatures (Dalziel et al. 2006).

FINAL CONCLUSIONS AND THE WAY FORWARD

Even though next generation sequencing methods are providing promising results, population structure analysis using traditional mitochondrial DNA markers will give the primary outline of phylogeography and produce a powerful baseline data. The present study is the most comprehensive genetic analysis of the *T. lepturus* populations across the Indian peninsular waters which revealed a significant genetic structuring across the marginal seas: Arabian Sea and Bay of Bengal at the mitochondrial DNA level. Our results suggest separate management protocols at to preserve and conserve the resource for the future. In India, where a multigear and multispecies fishery exists, limiting the exploitation throughout the stage of abundance to ensure the survival and replenishment of the stock will be a healthier stock management policy. Neutrality tests, mismatch analysis and bayesian skyline plots revealed a history of population expansion dating back to late Pleistocene epoch due to fluctuations in the sea level.

A major trouble associated with fisheries management is erroneous species identification. Taxonomic recognition of hairtails has always been a puzzle due to their similarity in body shape and coloration. Hence, the sequences deposited in the GenBank database will become an element of the common identification scheme and will help to resolve the ambiguities associated with the identification of species. High genetic differentiation among the samples of Southwest Atlantic Ocean, Pacific Ocean and Indian Ocean indicates cryptic speciation. Hence, a comprehensive morphological and genetic analysis to elucidate and confirm the species status of *Trichiurus* is suggested so that implementation of efficient management protocols can be done precisely.

Mitochondrial genomes are valuable for making inferences regarding population genetics, phylogenetics and recently in understanding adaptive variation in OXPHOS genes. Considering the importance, characterized the complete mitogenome of *T. lepturus* was characterized from Indian waters by PCR amplification and sequencing. The entire mitogenome is 16,840bp in length, with 13 protein coding genes, 2 subunit ribosomal RNA genes, and 1 control region similar to other vertebrates. Genome contains only 21 tRNAs ($tRNA^{Pro}$ is absent) as against 22 tRNAs reported in other vertebrates. The putative origin of L-strand replication (O_L) was located in a

cluster of five tRNA genes (WANCY region) and may not be functional due to the variation of conserved motif (5′-GCCGG-3′) and loop. These two unusual features are common for *Trichiurus* mitogenomes and can be used as a genus specific phylogenetic marker.

A phylogenetic analysis was conducted in order to reveal the inter-specific relationship of *T. lepturus* using mitochondrial PCGs and rDNAs. We also tried to resolve the intra and inter relationships of scombroid taxa for which the taxonomic classification remained contentious. As evidenced from the results, *T. lepturus* belongs to the Scombrodei family Trichiuridae and showed close relationship with *T. nanhaiensis* and *T. japonicus*. Monophyly of Trichiuridae was supported. Results confirm that the two families of billfishes are evolutionarily distinct from scombroids and should be placed in a separate suborder, Xiphioidei. The study also refutes the scombroid sister hypothesis due to the inclusion of several other percoid species between the billfishes and scombroids. Similar morphological traits and physiological adaptations allied with regional endothermy between scombroids and billfishes might have developed due to convergent evolution. Sphyraenidae which was earlier regarded as basal scombroid showed a closer relationship with Xiphioidei. Transcriptome and genome analysis should be conducted in future to provide evidence regarding the convergence of regional endothermy in distant teleost fishes.

Because elevated aerobic capacity is associated with the function of mitochondrial genes, further study was conducted to understand the pattern of selection acting upon OXPHOS genes that might have facilitated adaptive evolution in fast-moving fishes for their continuous swimming lifestyle. The study provides evidence for positive selection in teleost regional endotherms against a background of strong negative selection in the mitochondrial OXPHOS genes across several branches of the phylogeny. Changes found in the subunits ND5 and ND6 might have modified the proton pumping efficiency and assembly of complex I respectively and the substitutions found in the subunit ATP6 might have an impact on the rotor performance of the complex V. These mutations might have facilitated improvements in thermo genesis, metabolic potential, successful thermal niche expansion and further adaptation to the new environment for this group of species. Moreover, the results obtained provide exciting opportunities for further research on co-evolution of nuclear genes, physiology and various other aspects which will eventually help us to understand how a species can adapt to climate change in future.

PUBLISHED/ACCEPTED/COMUNICATED ARTICLES: 4

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Research guide

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